Efficient Knockout and Functional Analysis of BSSS-Related Genes by Episomal Expression Vector Containing Cas9 and AMA1 in Acremonium Chrysogenum

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Research Article

Keywords: Acremonium chrysogenum, cephalosporin C, CRISPR/Cas9, yeast bud site selection system

Posted Date: March 11th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1419582/v1

Abstract

Objective

The *sorB* gene related to sorbicillinoid production was used as the target, and the free expression element AMA1 was used to verify the availability of using this element in *Acremonium chrysogenum*.

Result

The point mutation of the *sorB* gene was successfully achieved in the Cripsr-Cas9 episomal expression system. In addition, the addition of *sorB* donor DNA in this system could achieve efficient, markless, and complete knockout of genes. Using this gene-editing platform, four BSSS-related genes Δ*axl1*, Δ*axl2*, Δ*bud3*, and Δ*bud4* were knocked out completely, and it was found that the yield, dry weight, and pH of the knockout strains did not change significantly. Further, the stress tolerance of the knockout strains was determined, and the relationship between morphology and stress tolerance was preliminarily analyzed.

Conclusion

The gene-editing efficiency exceeded 80% and the developmental process of arthrospores differed from the starting strain.

1. Introduction

The technology of CRISPR-Cas9 solved the long-term problems of low transformation efficiency and the large workload of screening mutant strains faced by filamentous fungi. In recent years, a variety of filamentous fungal gene-editing systems have been developed, such as in vivo and in vitro methods; different types of Cas proteins, vectors, or promoters, greatly enriched the control of gene expression and different needs in modified strains. Huang et al. (Huang et al. 2019) used Cas9 endonuclease (nCas) to achieve single-base gene editing and efficient knockout of orotidine-5'-decarboxylase pyrG, pigment gene *fwnA*, and non-phenotype gene *prtT*. However, the application of CRISPR-Cas9 in *A. chrysogenum was* still rare. For example, Chen et al. (Chen et al. 2020) constructed a U6/tRNA chimeric promoter and connected two sgRNAs to achieve efficient knockout of large gene fragments; Chu (Chen and Chu 2019) developed a simple CRISPR/Cas9 system for efficient disruption of *sorA* and *sorB* genes related to sorbicillinoid production. Both of these ways used integrated vectors which might increase the metabolic burden of the strains because of the uninterrupted expression of Cas9 and resistance gene. The autonomous and episomal replication of various plasmids containing AMA1 DNA fragments has been achieved in filamentous fungi such as *Aspergillus nidulans* (Gatherar et al. 2004), *Aspergillus fumigatus* (Khalaj et al. 2007), *Aspergillus niger* (Sarkari et al. 2017), *Penicillium chrysogenum* (Fierro et al. 1996), and *Monascus Purpureus* (Shimizu et al. 2006). However, there was no literature reported on the application of the episomal expression system in *A. chrysogenum*. Therefore, it is feasible to develop a

new episomal expressible system in *A. chrysogenum*, which would lead to less influence on the mutant strain and more reliable experimental results.

Biopigments are ubiquitous in fungi (Sharma and Meyer 2022) and the pigment coding gene is often used to determine gene editing efficiency due to their easily scored feature during screening when it is knocked out. The polyketide synthase *sorB*, as one of the genes in the sorbicillinoids synthesis-related gene cluster, knocked out the original yellow-colored strains to become colorless. When the mutation of the key gene cluster was repaired in *P. chrysogenum*, the strain recovered the activity of sorbicillinoids which had disappeared during the evolution process (Guzman-Chavez et al. 2017). Most of the high-yield cephalosporin C (CPC) strains of *A. chrysogenum* were colorless, which proved that these strains might be having mutations in the sorbicillinoids synthesis-related genes *sorA* or *sorB* (Zhgun and Eldarov 2021)

The production of CPC was considered to be closely related to unique arthrospores formation during fermentation in *A. chrysogenum*, but the molecular explanation of the relationship between them was still unclear. BSSS (yeast bud site selection system) was mainly composed of four proteins, Axl1, Axl2, Bud3, and Bud4, which were the landmark proteins of axial budding and mainly involved in the regulation of yeast axial budding. Similar to yeast, BSSS-related genes affected hyphae polar growth and septum formation in filamentous fungi. In *A. chrysogenum*, *Acaxl2* knockout accelerated arthrospore formation and recovered later in fermentation, and mutant strains had altered stress tolerance (Kluge and Kuck 2018). The four genes related to BSSS of *A. chrysogenum* were knocked out, respectively, in order to preliminarily explore the functions of these four genes on the process of morphogenesis and the growth state of these knockout strains under different stress conditions.

2. Materials And Methods

2.1 Microbial strains, and culture conditions

The strains and plasmids were listed in Table 1. *A. chrysogenum* CGMCC 3.3795, a wild-type strain, was purchased from the China General Microbiological Culture Collection Center (CGMCC). YPS (yeast extract peptone sucrose) medium and regeneration medium (Chen and Chu 2019) were used for protoplast preparation and transformation, respectively. The biomass was obtained by liquid culture before protoplast formation and protoplast regeneration after transformation, respectively. The transformation condition and process of *A. chrysogenum* were described previously (Chen and Chu 2019), with some modifications, and transformants were selected using media supplemented with hygromycin B (150 μg/ml) (Sangon Biotech, Shanghai China). Seed medium and fermentation medium (Xu et al. 2021) was used to detect characteristic parameters and morphological variation in fermentation process. *Escherichia coli* DH5α was employed for plasmid construction and grown for 24 h at 37℃ in Luria-Bertani (LB) medium containing 100 μg/mL ampicillin (Sangon Biotech, Shanghai China). *A. chrysogenum* and its derivatives were cultured on a malt extract solid medium for 10–14 days at 28℃ as previously described (Xu et al. 2021). Appropriate antibiotics (150 μg/ml hygromycin B) were added if required.

Table 1 is there.

2.2 Construction of plasmids and strains

All primer sequences were listed in Table 2 and the schematic diagram of the constructed plasmids is shown in Fig. 1a. The RGR (*Ribozyme-gRNA-Ribozyme*) fragment targeting *sorB*, also called pre-sgRNA (sorB), was generated using primers sgsorB-1, sgsorB-2, sgsorB-3, sgtarget-4 by overlapping PCR. The hygromycin fragment in pAN7-1 was replaced with pre-sgRNA (sorB), forming the plasmid pAN7-1-sorB. The PgpdA-pre-sgRNA (sorB)-TtrpC was amplified by primer pgpdA-F and trpC-R and template pAN7-1-sorB. The fragment was inserted into pFC332 which was cleaved by P*ac*I single enzyme with a one-step cloning kit (purchased from Vazyme, China), forming the plasmid pFC332-ΔsorB. The other four pre-sgRNA was obtained by the same method to replace the 20 bp protospacers (target gRNA). Each of them was inserted between *Bgl*II and *Stu*I sites within the pFC332-ΔsorB plasmid, forming the plasmid pFC332-Δtarget gene. Protospacers were designed by Cas-Designer, a web-based tool for selecting CRISPR-Cas9 target sites (Jeongbin et al. 2015).

Table 2 is there.

Transgenic *A. chrysogenum* produced by PEG-mediated plasmid uptake into protoplasts as described previously (Chen and Chu 2019), with some modifications. CRISPR/Cas9 editing system for base mutation was realized through mixing protoplasts and pFC332-Δtarget gene. With the addition of homology-directed repair template (donor DNA) during protoplast transformation, due to homologous recombination, large gene fragment deletion could be realized. The partial knockout of *sorB* and complete knockout of *axl1*, *axl2*, *bud3*, *and bud4* were accomplished. Each donor DNA was obtained by amplification and fusion of homologous left and right arms (Fig. 1a). The KOD One™ PCR Master Mix (TOYOBO CO., LTD. Life Science Department Osaka Japan) was used to perform colony PCR to verify the gene-edited transformant.

2.3 Fermentation and the determination of CPC titer

The inoculation amount, liquid volume in the flask, and culture condition for seed culture and fermentation process were described previously (Xu et al. 2021), with minor modification. The seed liquid which was cultured for 48 h at 28℃ was mixed with 50% (v/v) glycerol in the ratio of 1:1 and stored at −80℃. When needed, the seed liquid was inoculated into the seed medium and then transferred to the fermentation medium. After centrifugation and filtration of fermentation broth, the yield of CPC was determined using high-performance liquid chromatography (HPLC), as described previously in the details (Xu et al. 2021). Cephalosporin C sodium salt for biological product reference standards was purchased from Shanghai Macklin Biochemical Co., Ltd.

2.4 Determination of cell morphology and the ratio of arthrospore number

After the end-of-fermentation sample was centrifuged, 2.5% (v/v) glutaraldehyde was used to resuspend the precipitation and fix it overnight at 4°C. Phosphate buffer (Sangon Biotech, Shanghai China) was used to wash the sample for three times. The method for calculating the ratio of arthrospores was consistent with that reported by Xu et al. (Xu et al. 2021) The numbers of hyphae that did not swell and arthrospores were counted as a and b, respectively, and the swollen hyphae and free arthrospores were regarded as arthrospores. The formula for the ratio of arthrospores number was: arthrospores%= b/(a + b). The number of hyphae analyzed at each time point was never less than 150. The morphology of hyphae and arthrospores were observed using an M31 biological microscope which was purchased from Guangzhou Micro-shot Optical Technology Co., Ltd. Images were acquired with an MS60 camera and MShot Image Analysis System. Image editing was performed using Adobe Photoshop 2020.

For scanning electron microscopy, the mycelial suspension after washing with PBS was dehydrated step by step using 30%, 50%, 70%, 90% (v/v) ethanol, and finally, 100% ethanol was dehydrated twice, as described previously (Zhang et al. 2018). After vacuum drying and spray gold, the morphology of hyphae and arthrospores was observed under a Hitachi S-3400N scanning electron microscope. All assays were performed in triplicate.

2.5 Stress-dependent growth assay

The tolerance experiments of strains under different stress conditions refer to Kluge and Kuck (Kluge and Kuck 2018), with some modifications. 2 mL of MM (minimal medium) was added to small 30 mm Petri dishes to quantify the medium content. After mycelium of the same wet weight was resuspended in sterile water, 2 µl of each *A. chrysogenum* mycelium suspension was inoculated and grown on the solid medium for 7 days at 28°C. The effects of ER stress were tested using 5, 10 mM DTT. The effects of cell wall stress were tested using 20, 50, 100 mg/L CFW and 0.01, 0.03, 0.05% (w/v) Congo red. The effects of osmotic stress were tested using 0.5, 1, 1.5 M of NaCl, KCl, glucose, and sorbitol, respectively. All assays were performed in triplicate.

3. Results

3.1 Gene identification of *sorB* and gene involved in the BSSS

Based on sequence alignment, the sequence similarity of the *sorB* and four BSSS related gene (*axl1*, *axl2*, *bud3*, *bud4*) from CGMCC 3.3795 to the sequence of ATCC 11550 was 99.99%, 99.99%, 100%, 100% and 100% at the nucleotide level, with the GenBank accession numbers ACRE_048170, ACRE_012750, ACRE_053550, ACRE_040160, and ACRE_002510, respectively.

3.2 Validation of *sorB* gene point mutation and BSSS-related gene knockout strains

The *sorB* knockout strain changed from yellow to white due to a lack of pigment production in *A. chrysogenum* (Fig. 1b). The mutation sites of Δ*sorBa* were verified by PCR, and it was found that the mutation sites of all transformants were deletions at sites 4–5 of the gRNA (Fig. 1b), which reflected the conservative type of point mutation using this system. However, if donor DNA was added during the transformation process, it was not strictly homologous recombination, and uncertain sequence changes may occur in the process of complete gene knockout.

Figure 1 **is there.**

The construction process of plasmid pFC332-Δtarget and amplification of donor DNA were shown in Fig. 2a and Fig. 2b, respectively. PCR validation (Fig. 2c), indicates that the four BSSS related genes were successfully knocked out. The specific electrophoresis verification process was shown in the legend of Fig. 2.

Figure 2 **is there.**

3.3 Gene editing efficiency and homology arm length analysis

The sgRNAs of five genes (*sorB*, *axl1*, *axl2*, *bud3*, and *bud4*) were all selected near the start of the open reading frame. The gene lengths of the five genes, the lengths of the left and right arms of homology, the number of transformants, and the editing efficiency were shown in Table 3. The length of the knockout gene and the length of the homologous left and right arms did not affect the number of transformants and gene editing efficiency. The length of the left and right arms of 600–1000 bp could complete the homologous recombination of *A. chrysogenum*, and the editing efficiency exceeded 80%. The efficient gene editing greatly reduced the screening workload. The gene length statistics of five genes showed that the system could efficiently knock out gene fragments of 2–10 kb.

Table 3 is there.

3.4 CPC production and dry weight of mycelia

The relatively lower CPC production in the knockout mutants (four genes associated with BSSS) during fermentation compared with the control was recovered at the fermentation ends, except that Δ*axl1* production remained slightly decreased. In *A. chrysogenum* A3/2, Δ*axl2* did not affect CPC yield (Kluge and Kuck 2018), which was consistent with the results of this experiment. But Δ*axl2* significantly increased CPC production almost three times higher than that of the original industrial strain FC^3-5-23 (Xu et al. 2021), which reflected the difference between industrial and wild strains in the case of the same genetic mutation. The same dry weight of mycelia during fermentation indicated that the knockout of these genes did not affect cell growth (Fig. 3b). Furthermore, the pH of these knockout strains at the fermentation ends was not significantly changed compared with the starting strain (Fig. 3c).

Figure 3 **is there.**

3.5 Mycelial morphology and arthrospore ratio

As shown in Fig. 3d, the arthrospore ratio of each strain, either the starting strain or the mutant strain, continued to increase with fermentation time. Compared with the wild strain CGMCC 3.3795, the number of arthrospores in the four knockout strains was different to some extent. Specifically, Δ*axl1* decreased the number of arthrospores in the fermentation process, while the formation of arthrospores became faster in Δ*axl2*, Δ*bud3*, and Δ*bud4*, especially at 48 and 72 h. But at 96 h, CGMCC 3.3795 arthrospores formed rapidly, which was comparable to the proportion of arthrospores among the three mutation strains. At the end of fermentation, except for Δ*axl1*, which arthrospores ratio was 75.85%, the arthrospores of the other four strains all reached or approached to 90%. Figure 4 showed the morphology of hyphae in different shake flask fermentation periods. It could be found that the wild strain began to form free arthrospores at 72 h of fermentation. In contrast, the free arthrospores formation of *Δaxl1* lagged behind, until 108 h, and free arthrospores in Δ*axl2* and Δ*bud3* strains appeared earlier, at approximately 48 h.

Figure 4 **is there.**

Compared with CGMCC 3.3795, more "arthrospore strings" were formed in Δ*axl1* at 48 h and 72 h. Except for Δ*axl1*, the morphology of fermentation ends in other strains were all composed of a large number of free arthrospores and a small number of hyphae. In addition, the morphological observation of the hyphae and arthrospores of the wild strain and the Δ*bud3* strain by scanning electron microscope (Fig. 5) showed that the diameters and shape of the hyphae were similar. The arthrospores were ellipsoid in shape, similar in size, the surface was flat as a whole, and some arthrospores were slightly wrinkled. In general, there was no significant difference between the two strains.

Figure 5 **is there.**

3.6 Stress tolerance

Axl2 was involved in the control of the endoplasmic reticulum, cell wall, and osmotic stress (Kluge and Kuck 2018). As shown in Fig. 6, the changes in stress tolerance of strains with the knockout of four BSSS-related genes were explored. DTT was used to explore the endoplasmic reticulum stress level of the strain. Figure 6a showed that Δ*axl2*, Δ*bud3*, and Δ*bud4* slightly increased the endoplasmic reticulum stress level compared with the starting strain CGMCC 3.3795. In the case of 10 mM DTT addition, the Δ*axl1* strain turned yellow, which was suggested that the Δ*axl1* mutation strain produced more yellow pigment under this condition. Congo red and fluorescent white were used to explore the cell wall stress level of the strains. Figure 6b showed that the colony diameter of the four knockout strains was smaller in the MM medium with high concentrations of Congo red and fluorescent white, suggesting the level of cell wall stress declined in the knockout strains. NaCl, KCl, glucose, and sorbitol were used to explore the osmotic stress level of the strain. Figure 6c and Fig. 6d showed that although the colony diameter of the knockout strain did not change significantly, the yellowness of the colony was different. Specifically, under the conditions of glucose addition, the yellow degree of Δ*axl1* mutation strain colonies was

significantly deeper than that of wild strain and other knockout strains. In addition, under the addition of 1 and 1.5M KCl, the yellowness of the Δ*bud3* colony was significantly deeper than that of the wild strain; under the addition of 1.5M NaCl, the yellowness of Δ*bud3* and Δ*bud4* was significantly deeper than that of the wild strain.

Figure 6 **is there.**

4. Discussion

The discovery of the AMA1 sequence derived from *Aspergillus nidulans* was the only element reported that could replicate autonomously in *Aspergillus*, and its discovery enabled the episomal expression of plasmids in filamentous fungi. The efficient utilization of this sequence in the plasmid overcame the metabolic burden of mutation strains, caused by the integration of the overexpressed gene into the genome, especially when the Crispr-Cas9 system was employed, the continuous expression of Cas9 was unnecessary. The plasmid pAN7-1-sorB constructed by Chen (Chen and Chu 2019) contains multiple PgpdA promoters, which may affect the integration process and the stability of the integrated fragment in the genome. In addition, we also tried to replace the Cas9 expression cassette and hygromycin expression cassette in pFC332 with the original parts in pAN7-1-sorB to construct a new integrated expression plasmid, but its point mutation efficiency was far less than that of this newly constructed system.

PCR validation on the *sorB* point mutant strain showed that the mutation sites of all transformants were deletions at sites 4–5 of the gRNA (Fig. 1b), and the conserved mutation sites could be used for a more accurate gene-editing process. The selection of the left and right arms of the five target genes was between 600 and 1000 bp, and the length of the homology arm had no obvious effect on the gene-editing efficiency. During homologous recombination in filamentous fungi, the length of the homology arm was usually chosen to be around 1 kb. In *Aspergillus niger*, homologous recombination could be accomplished even if the homology arm length was only 39 bp (Dong et al. 2019). Whether this system could use shorter left and right arms to achieve homologous recombination remains to be further verified in *A. chrysogenum*.

The special morphogenesis process of filamentous fungi was considered to be closely related to the metabolic process. During the fermentation process of *A. niger*, the morphology of hyphal was more favorable to the production of enzymes, and the uniform mycelial spherical shape was more favorable for the production of citric acid (Papagianni 2004). There was a certain correlation between the production of CPC and the formation of arthrospores in *A. chrysogenum*, and the overexpression of the *axl2* gene of the industrial strain FC^3-5-23 significantly affected the proportion of arthrospores and the yield of CPC (Xu et al. 2021). However, the knockout of the four genes Δ*axl1*, Δ*axl2*, Δ*bud3*, and Δ*bud4* in the wild strain CGMCC 3.3795, which affected the formation of arthrospores, did not significantly affect the formation of CPC. This result might suggest that the other limiting factors rather than morphology hindered the CPC production in the wild strain, so even if the morphology of knockout strains had been changed, the yield of CPC had little impact. In fact, we also identified the key gene for valine production

(acetolactate synthase, GME5735_g, homologous to ACRE_004720 in ATCC 11550) and key genes in the PPP pathway involved NADPH formation (Glucose-6-phosphate 1-dehydrogenase: GME3969_g homologous to ACRE_033950 in ATCC 11550; 6-phosphogluconate dehydrogenase: GME8565_g and GME7826_g, homologous to ACRE_056380 in ATCC 11550 and XM_018280118.1 in *Pochonia chlamydosporia 170*, respectively in the high-yielding strain FC^3-5-23 (whole genome sequenced, unpublished). Overexpression of these key genes in the wild strain CGMCC 3.3795 can increase the CPC production. This phenomenon suggested that if we anticipate to increase the CPC production of CGMCC 3.3795, we need to start from the perspective of global metabolic engineering rather than morphological regulation. This study also tried to measure the oxidative stress ability of the knockout strain and found that neither the starting strain nor the knockout strains could tolerate 0.0025% (v/v) hydrogen peroxide. 1 μL 10^4 CGMCC 3.3795 spores could not grow on 1.8mM (0.0055%) hydrogen peroxide plates. This phenomenon was different from *A. chrysogenum* A3/2 (Kluge and Kuck 2018) and industrial production strain FC^3-5-23 (Xu et al. 2021). Both of them could grow on the MM medium containing 0.0075% (v/v) hydrogen peroxide, indicating that the oxidative stress ability of *A. chrysogenum* CGMCC 3.3795 was relatively weak compared with other reported strains.

The morphological changes of hyphae and the changes of stress response in the knockout strains of *axl2, bud3*, and *bud4* were similar, indicating that the gene functions of the three genes were similar. The Δ*axl1* strain showed higher degrees of yellow color in the colonies under ER and osmotic pressure conditions, suggesting that although Δ*axl1* did not increase CPC production, it might increase other secondary metabolites such as yellow pigment sorbicillinoids under certain stress conditions. Although there was a correlation between the morphology and the antioxidant capacity of the strains, the growth and stress resistance levels of different strains after knocking out the same gene were different due to the differences in the growth, morphogenesis process, and the capacity of stress tolerance.

5. Conclusions

The yellow pigment sorbicillinoids synthesis-related gene *sorB* was used as the target to establish a high-efficiency gene knockout system for free expression of Cas9 in CGMCC 3.3795 for the first time, so as to reduce the metabolic burden caused by the integration of Cas9 into the genome. Subsequently, four BSSS-related genes *axl1*, *axl2*, *bud3*, and *bud4* were knocked out with this gene-editing system. Compared with the starting strain CGMCC 3.379, the knockout strain had no significant effect on CPC yield, hyphal dry weight, and pH. In addition, Δ*axl1* caused a continuous decrease in the proportion of arthrospores, while arthrospores formation were earlier in Δ*axl2*, Δ*bud3*, and Δ*bud4*, and the proportion of arthrospores at the end of fermentation was similar to that of the wild strain CGMCC 3.3795, which were all around 90%. Through stress tolerance experiments, it was found that compared with the starting strain CGMCC 3.3795, Δ*axl1*, Δ*axl2*, Δ*bud3*, and Δ*bud4* had similar changes in colony growth state under different stress conditions. Besides, Δ*axl1* produced more yellow pigment than other strains under different endoplasmic reticulum and osmotic pressure conditions.

Declarations

Acknowledgments

This work was financially supported by the National Key Research Development Program of China (No. 2019YFA0904800).

Author Contribution Statement

Liu Land Chu Jdesigned research. Liu L and Chen Z conducted experiments and Liu L analyzed data and wrote the manuscript. Chu J and Tian XW revised the manuscript. All the authors read and approved the final manuscript.

Conflict of interest

The authors declare no competing financial interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Tables

Tables 1, 2 and 3 are not available with this version

Figures

Figure 1

Construction of CRISPR-Cas9 knockout system containing AMA1 in *A. chrysogenum*. A: knockout plasmids and donor DNA; b: *sorB* mutation site and *sorB* knockout strain morphology.

Figure 2

Plasmid construction process and PCR validation of knockout strains. (a) The amplification of left and right arm fragments and fusion PCR. 1: *axl1* left arm fragment (968bp); 2: *axl1* right arm fragment (980); 3; overlap PCR fragments of *axl1* left and right arm fragments (1948bp);

4: *axl2* left arm fragment (724bp); 5: *axl2* right arm fragment (682bp); 6; overlap PCR fragments of *axl2* left and right arm fragments (1406bp); 7: *bud3* left arm fragment (674bp); 8: *bud3* right arm fragment (834bp); 9; overlap PCR fragments of *bud3* left and right arm fragments (1508bp); 10: *bud4* left arm fragment (785bp); 11: *bud4* right arm fragment (735bp); 12; overlap PCR fragments of *bud4* left and right arm fragments (1520bp); M: DL 5000 Marker; 13: *sorB* left arm fragment (1049bp); 14: *sorB* right arm fragment (982bp); 15; overlap PCR fragments of *sorB* left and right arm fragments (2031bp); (b) The electrophoretogram of pFC322-Δtarget construction process. M1: DL 500 Marker; 1: sg12 (86bp); 2:sg34 (108 bp); 3: sg14 (294 bp, overlap PCR fragments of sg12 and sg34); M2: DL 2000 Marker; M3: DL 15000 Marker; 4: PFC332-ΔsorB; 5: PFC332-ΔsorB digested by *Bgl*II and *Stu*I; (c) PCR validation of knockout strains. M: DL 10000 Marker; 1: positive control of *sorB* (2031 bp); 2: PCR validation of PFC332-ΔsorB using primers of axl1-UH-F and axl1-DH-R; 3: positive control of upstream and downstream of *sorB* (2031 bp); 2: PCR validation of PFC332-ΔsorB using primers of sorB-UH-F and sorB-DH-R (2031 bp); 3: positive control of upstream and downstream of *axl1* (1948 bp); 4: PCR validation of PFC332-Δaxl1 using primers of axl1-UH-F and axl1-DH-R (1948 bp); 5: positive control of upstream and downstream of *axl2* (1406 bp); 6: negative control of upstream and downstream of *axl2* (The genome of CGMCC 3.3795 as template, 4049 bp); 7: PCR validation of PFC332-Δaxl2 using primers of axl2-UH-F and axl2-DH-R (1406 bp); 8: positive control of upstream and downstream of *bud3* (1508 bp); 9: negative control of upstream and downstream of *bud3* (The genome of CGMCC 3.3795 as template, 6069 bp); 10: PCR validation of PFC332-Δbud3 using primers of bud3-UH-F and bud3-DH-R (1508 bp); 11: positive control of upstream and downstream of *bud4* (1520 bp); 12: negative control of upstream and downstream of *bud4* (The genome of CGMCC 3.3795 as template, 6032 bp); 13: PCR validation of PFC332-Δbud4 using primers of bud4-UH-F and bud4-DH-R (1520 bp);

Figure 3

Shake flask fermentation parameters and arthrospore ratio of four BSSS related gene knockout strains. a: CPC production; b: mycelium dry weight; c: pH; d: the proportion of arthrospores in all hyphal forms. Data represent the average values and standard deviations from three replicates.

Figure 4

Morphology of wild-type strain CGMCC 3.3795 and four BSSS related gene knockout strains at different fermentation time points.

Figure 5

Morphology of hyphae and arthrospores of wild strain CGMCC 3.3795 and *bud3* knockout strain under the scanning electron microscope

Figure 6

Growth of wild-type strain CGMCC 3.3795 and four BSSS related gene knockout strains in MM medium under different stress conditions. a: ER stress-related growth (MM and 5、10 mM DTT); b: Cell wall and stress-related growth (0.01、0.03、0.05% (w/v) Congo red and 20、50、100 mg/L Calcofluor white); c: Osmotic stress-related growth (0.5、1、1.5 M NaCl and KCl); c: Osmotic stress-related and high concentration of sugar growth (0.5、1、1.5 M glucose and sorbitol). Growth tests were assayed in triplicate on minimal medium (MM) which served as the control without any stressor.

Supplementary Files

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- SupplementaryMaterials.docx